

# Abundance of six tetracycline resistance genes in wastewater lagoons at cattle feedlots with different antibiotic use strategies

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## Summary

The abundance of six tetracycline resistance genes *tet(O)*, *tet(Q)*, *tet(W)*, *tet(M)*, *tet(B)* and *tet(L)*, were quantified over time in wastewater lagoons at concentrated animal feeding operations (CAFO) to assess how feedlot operation affects resistance genes in downstream surface waters. Eight lagoons at five cattle feedlots in the Midwestern United States were monitored for 6 months. Resistance and 16S-rRNA gene abundances were quantified using real-time PCR, and physicochemical lagoon conditions, tetracycline levels, and other factors (e.g. feedlot size and weather conditions) were monitored over time. Lagoons were sorted according to antibiotic use practice at each site, and designated as 'no-use', 'mixed-use' or 'high-use' for comparison. High-use lagoons had significantly higher detected resistance gene levels ( $tet^R$ ;  $2.8 \times 10^6$  copies ml<sup>-1</sup>) relative to no-use lagoons ( $5.1 \times 10^3$  copies ml<sup>-1</sup>;  $P < 0.01$ ) and mixed-use lagoons ( $7.3 \times 10^5$  copies ml<sup>-1</sup>;  $P = 0.076$ ). Bivariate correlation analysis on pooled data ( $n = 54$ ) confirmed that  $tet^R$  level strongly correlated with feedlot area ( $r = 0.67$ ,  $P < 0.01$ ) and 'total' bacterial 16S-rRNA gene level in each lagoon ( $r = 0.51$ ,  $P <$

0.01), which are both characteristic of large CAFOs. *tet(M)* was the most commonly detected gene, both in absolute number and normalized to 16S-rRNA gene level, although *tet(O)*, *tet(Q)* and *tet(W)* levels were also high in the mixed and high-use lagoons. Finally, resistance gene levels were highly seasonal with abundances being 10–100 times greater in the autumn versus the summer. Results show that antibiotic use strategy strongly affects both the abundance and seasonal distribution of resistance genes in associated lagoons, which has implications on water quality and feedlot management practices.

## Introduction

Growing evidence exists that antibiotic use in agriculture is affecting antibiotic resistance in human pathogens via the food supply (Witte, 1998; Angulo *et al.*, 2000; UCS, 2001; Iovine and Blaser, 2004; Molbak, 2004; Wang *et al.*, 2006); however, similar impacts through the water supply are less well established. For example, antibiotic application in concentrated animal feeding operations (CAFOs) may transfer 'resistance' via hydrologic processes below the point of use and have environmental or public health consequences. In fact, Chee-Sanford and colleagues (2001) found tetracycline resistance genes up to 250 m away from the point of use in groundwater and Smith and colleagues (2004) found a weak correlation between resistance gene numbers and tetracycline levels in feedlot lagoons, presumptively via overland flow. The purpose of this study was to holistically assess factors that affect the abundance and seasonality of released resistance genes in surface water below CAFOs, especially related to on-site CAFO operating practices.

As background, the National Committee for Clinical Laboratory Standards (NCCLS) defined four use categories for antibiotics in livestock (NCCLS, 1999): 'therapeutic use' on animals that exhibit clinical disease; 'control use' on herds where the morbidity and/or mortality has exceeded an accepted baseline; 'prevention-prophylaxis use' on exposed healthy animals to minimize the onset of disease; and 'growth promotion' where antibiotics are used as additives to improve physiologic performance of the animals. Although these uses are distinct, each

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**Table 1.** Descriptions of the eight feedlot lagoons monitored in the sampling program.

Feedlot	Feedlot capacity <sup>a</sup>	Lagoon	Average head per lagoon <sup>b</sup>	Approximate lagoon size (m <sup>2</sup> ) <sup>c</sup>	Tet use	Additional notes
MR	15 000	MR1	2 000	1.0 × 10 <sup>4</sup>	Mixed-use	Cattle penned separately and receive no tetracycline; cattle needing treatment removed from pens.
		MR2	3 000	1.5 × 10 <sup>4</sup>	Mixed-use	Collects water from all lagoons on feedlot.
O1	2 000	O1	110	1.2 × 10 <sup>2</sup>	No-use	Extensive feed management plan. No tetracycline use.
O2	12 000	O2	470	6.2 × 10 <sup>3</sup>	No-use	Feedlot depopulates over winter months. No tetracycline use.
UA	27 000	UA1	6 400	1.0 × 10 <sup>4</sup>	High-use	Lagoon drains hospital and receipt pens. Water used to irrigate fields.
		UA2	1 000	2.1 × 10 <sup>3</sup>	High-use	Cattle are rotated through pen areas; lagoon drains pens with tetracycline use.
UB	34 000	UB1	630	1.5 × 10 <sup>2</sup>	High-use	Pens drain into lagoon, but lagoon often pumped to low water levels.
		UB2	33 180	2.6 × 10 <sup>4</sup>	High-use	Lagoon is the last unit in a lagoon series. Lagoon used to irrigate fields.

a. The maximum feedlot capacity.

b. The average head of cattle directly or indirectly draining into the lagoon.

c. A gross approximation of lagoon surface area. Surface area was calculated from provided maps or by comparison to similar lagoons.

feedlot has its own antibiotic strategy, which varies depending upon the purpose, timing, amount and type of antibiotic used. Regardless of specific local strategies, antibiotic use at feedlots generally follows one of three basic regimes: 'no-use' operations (organic or quasi-organic feedlots); 'mixed-use' operations that use antibiotics for sick or quarantined animals; or 'high-use' operations, where antibiotics are used for some or all of the NCCLS applications. Unfortunately, feedlots are rarely 'public' about antibiotic use or even keep close records (Webb and Davies, 1993); therefore, it has always been difficult to link specific antibiotic use practices and resistance-related impacts.

The goal of this project was to examine how CAFO practices affect the absolute and relative resistance gene abundances downstream of CAFO operations. Five feedlots reflecting no-use, mixed-use and high-use approaches were chosen for evaluation and eight associated wastewater lagoons were monitored for 6 months to examine resistance gene levels over time. Tetracycline was chosen for study because it and its derivatives are used for most of NCCLS applications; the genetic basis of tetracycline resistance is well established, permitting 'resistance' detection using molecular methods (Chopra and Roberts, 2001); and specific uses at site each were quite well established. The resistance genes quantified (using real-time PCR) were *tet(O)*, *tet(Q)*, *tet(W)*, *tet(M)*, *tet(L)* and *tet(B)*, which were chosen because they all have been detected at CAFOs; they reflect ribosomal and efflux antibiotic mechanisms; and they have established associations with bacteria of public health interest (Scott *et al.*, 2000; Aminov *et al.*, 2001; Chopra and Roberts, 2001; Roberts, 2005). Further, the sum of this gene-set (*tet<sup>R</sup>*) previously correlated with whole-organism plate counts of resistant organisms in lagoons (Smith *et al.*,

2004). Because each CAFO operation was unique, physicochemical lagoon and weather conditions were monitored to assess how such factors might influence resistance gene levels and patterns at each site.

## Results

### *Feedlots and lagoons in the study*

Descriptions and designations of the five feedlots and eight lagoons used in this study are provided in Table 1. The feedlots were all within about 700 km of each other, and capacities ranged from 2000 to 34 000 head of cattle. The lagoons ranged in surface area from 1.2 × 10<sup>2</sup> to 2.6 × 10<sup>4</sup> m<sup>2</sup>. Unfortunately, no quantitative data for tetracycline use were available for any feedlot (except the no-use lots); therefore, lagoons were sorted into three groups based on interviews with each feedlot operator and personal on-site investigations.

Feedlots O1 and O2 had single lagoons and were defined as 'no-use' because antibiotics were not used except on animals removed from the herd. Feedlot MR had two lagoons, MR1 and MR2, and was designated as 'mixed-use' because tetracycline was used therapeutically or had been used for other purposes within the last 5 years. Feedlots UA and UB had two monitored lagoons each (UA1, UA2, UB1 and UB2), and were designated 'high-use' because tetracycline(s) were used for various NCCLS applications at the sites.

### *Physical and chemical water conditions in the lagoons*

Mean physical and chemical water conditions, water-column tetracycline levels, and weather-related parameters associated with each lagoon are provided in Table 2.

**Table 2.** The sample mean chemical conditions of each sample lagoon.

Lagoon	Tetracycline ( $\mu\text{g l}^{-1}$ )	TSS <sup>a</sup> ( $\text{mg l}^{-1}$ )	VSS ( $\text{mg l}^{-1}$ )	Water temperature ( $^{\circ}\text{C}$ )	pH	Dissolved oxygen ( $\text{mg l}^{-1}$ )	Air Tmax <sup>b</sup> ( $^{\circ}\text{C}$ )	Air Tmin ( $^{\circ}\text{C}$ )	Total precip. (cm)	Total sunlight <sup>c</sup> (Hours)
MR1	0.45 (0.31) <sup>d</sup>	3 800 (2 600)	1 400 (850)	18.8 (4.4)	7.72 (0.19)	0.05 (0.08)	26.7 (3.0)	11.1 (4.1)	5.7 (4.6)	13:08 (1:07)
MR2	4.13 (2.77)	5 500 (3 600)	1 700 (950)	18.6 (5.7)	7.61 (0.22)	0.15 (0.29)	26.7 (3.0)	11.1 (4.1)	5.7 (4.6)	13:08 (1:07)
O1	0.95 (0.62)	3 500 (3 300)	1 300 (1 200)	22.2 (6.5)	7.96 (0.20)	0.37 (0.36)	25.7 (3.1)	10.9 (4.1)	3.9 (3.0)	13:07 (1:06)
O2	0.71 (0.61)	7 800 (4 400)	2 300 (1 100)	20.6 (5.3)	7.96 (0.14)	0.96 (1.53)	29.7 (3.1)	10.9 (4.1)	1.9 (2.0)	13:01 (1:02)
UA1	10.7 (3.69)	11 000 (6 400)	4 000 (2 500)	20.1 (3.9)	7.98 (0.19)	0.34 (0.52)	29.8 (3.7)	14.8 (3.8)	1.9 (1.3)	12:58 (0:59)
UA2	16.4 (1.94)	17 000 (6 400)	6 600 (3 100)	18.7 (1.9)	7.85 (0.10)	0.07 (0.09)	29.8 (3.7)	14.8 (3.8)	1.9 (1.3)	12:58 (0:59)
UB1	5.04 (2.87)	23 000 (9 600)	12 000 (3 100)	20.1 (4.3)	7.41 (0.24)	0.01 (0.01)	28.2 (3.9)	13.7 (4.1)	3.7 (4.1)	12:32 (1:04)
UB2	2.06 (1.16)	1 900 (1 100)	800 (350)	21.9 (3.8)	7.82 (0.20)	0.10 (0.15)	28.2 (3.9)	13.7 (4.1)	3.7 (4.1)	12:57 (0:57)

a. TSS, VSS, water temperature, pH and DO are mean values based on eight separate samples.

b. Air Tmax, Tmin and total precipitation are mean values based on the time period between each lagoon sampling.

c. Total sunlight means based on time period between each lagoon sampling.

d. 95% CI provided in brackets.

Statistical analysis was performed on mean water conditions and weather data at each lagoon, and for lagoons grouped under each use strategy. Only tetracycline level, total suspended solids (TSS), volatile suspended solids (VSS), and pH differed significantly among the eight lagoons (Kruskal–Wallis;  $P < 0.05$ ). Except for tetracycline level ( $P < 0.05$ ), conditions were statistically identical in the no-use and mixed-use lagoons, therefore all significant differences among lagoons were seen at high-use facilities.

Table 3 compares mean levels of tetracycline, VSS, TSS and 16S-rRNA genes for the three types of lagoons. Similar to individual lagoon data, all four parameters were significantly higher in high-use lagoons relative to the other lagoons (Kruskal–Wallis;  $P < 0.01$ ). Elevated VSS and 16S-rRNA gene levels in the high-use lagoons are noteworthy because they indicate that background biosolids and bacterial community abundances, respectively, are higher in those lagoons. As such, both absolute resistance gene numbers and gene numbers normalized to 16S-rRNA gene level were quantified to verify that observed higher resistance gene numbers in the high-use

lagoons were not simply a reflection of larger microbial communities.

#### *Resistance gene copy numbers versus tetracycline use strategy*

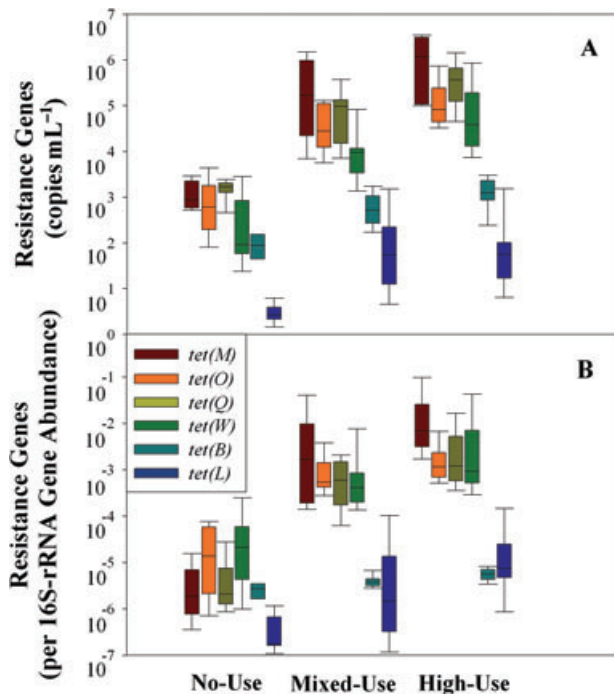
Absolute abundances of all six genes in the lagoons increased as presumed antibiotic use increased (Fig. 1A), although *tet(M)*, *tet(Q)*, *tet(O)* and *tet(W)* increased most dramatically at the high-use feedlots. All four genes were 100–1000 times greater in the mixed- and high-use feedlot lagoons compared with no-use lagoons (Kruskal–Wallis;  $P < 0.01$ ), and 10–100 times greater in the high-use versus the mixed use lagoons, although this second difference was not statistically significant (Kruskal–Wallis;  $P = 0.21$ ). *tet(B)* and *tet(L)* levels increased slightly associated with higher antibiotic use, although detected levels were much lower than the other four genes.

To segregate the impact of differing biosolids levels among the lagoons, the absolute gene data were normalized to 16S-rRNA gene level to determine the abundance

Antibiotic use strategy	Tetracyclines ( $\mu\text{g l}^{-1}$ )	TSS ( $\text{mg l}^{-1}$ )	VSS ( $\text{mg l}^{-1}$ )	16S-rRNA gene ( $\text{ng ml}^{-1}$ )
No-use	0.83 (0.43)	5 700 (2 900)	1800 (830)	$1.7 \times 10^7$ ( $7.8 \times 10^6$ )
Mixed-use	2.3 (1.6)	4 600 (2 200)	1600 (620)	$2.4 \times 10^7$ ( $1.2 \times 10^7$ )
High-use	8.8 (2.4)	12 000 (5 100)	5300 (2400)	$6.1 \times 10^7$ ( $4.5 \times 10^7$ )

**Table 3.** Mean water column tetracycline and 16S-rRNA gene levels for lagoons associated with the three antibiotic use strategies.

Brackets denote 95% CI.



**Fig. 1.** Tetracycline resistance gene copy numbers of *tet(O)*, *tet(W)*, *tet(Q)*, *tet(M)*, *tet(L)* and *tet(B)* in the no-use, mixed-use and high-use feedlot lagoons: (A) Absolute concentrations of each gene and (B) gene abundances normalized to ambient 16S-rRNA gene level. Boxes refer to the span of the 25th and 75th percentiles of the data, the line is the median value, and error bars refer to 95% CI.

of resistance genes relative to the whole bacterial community. The same general patterns in gene abundance were seen in the normalized data (Fig. 1B) relative to absolute data (Fig. 1A), although data indicate that abundances of *tet(B)* and *tet(L)* were only minimally affected by antibiotic use practice.

#### Correlations between resistance gene copy numbers and operating conditions

A bivariate correlation analysis was performed between  $tet^R$ , and measured physicochemical lagoon data, weather conditions, and operational factors at each site to assess the influence of other environmental factors on absolute resistance gene abundances (Table 4,  $P < 0.05$ ). The strongest positive correlations with  $tet^R$  were lot size (i.e. feedlot area) and 16S-rRNA gene level ( $r = 0.67$  and  $0.51$  respectively), whereas the strongest negative correlations were with length of day and sunlight level at each site ( $r = -0.40$  and  $-0.39$  respectively). Large lot sizes and higher 16S-rRNA gene levels are both characteristic of high-use facilities and are consistent with data in Fig. 1. Interestingly, only a weak correlation was observed between  $tet^R$  and tetracycline level. The significant negative correlation between  $tet^R$  and sunlight hours

is particularly interesting because recent data have shown that elevated light exposure can increase disappearance rates of resistance genes in aquatic systems (Engemann *et al.*, 2006).

#### Temporal changes in lagoon resistance gene copy numbers

Figure 2 presents individual gene data and  $tet^R$  abundances over time in the lagoons for absolute and normalized data. Figure 2B, C, E and F shows that both mixed-use and high-use feedlot lagoons had large increases in resistance gene levels in the autumn samples, whereas Fig. 2A and D indicates that no consistent temporal trends existed in resistance gene levels in the no-use lagoons. Normalized gene abundances varied more over time than non-normalized abundances in no-use lagoons; however, no-use lagoon abundances were always low compared with the higher-use systems. These data broadly imply that any tetracycline use at a site can influence both the absolute abundance and seasonal levels of resistance genes at a given feedlot, although the most dramatic temporal changes in abundances were observed in the high-use lagoons, where levels were 10–100 greater in autumn versus summer samples.

Although statistics are not provided, *tet(M)* increased dramatically in the mixed- and high-use autumn samples, suggesting that *tet(M)* was most affected by the manner of use or transport of tetracycline at the higher-use facilities. This is most dramatically seen in Fig. 2E and F where *tet(M)* copy number represents nearly 20% of the copy numbers of 16S-rRNA genes in the lagoons.

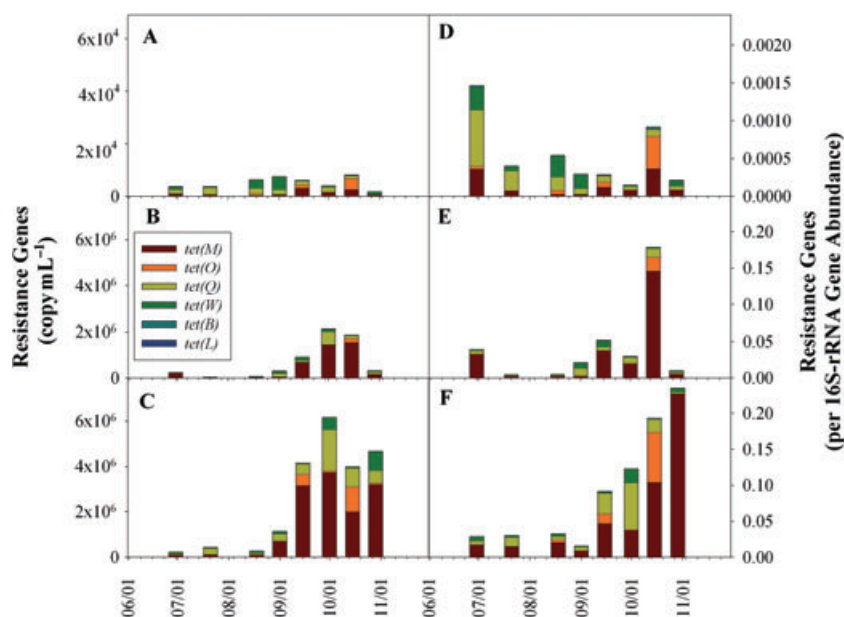
## Discussion

Considerable concern exists regarding the high use of antibiotics in agriculture, especially antibiotics that might confer resistance to human and other pathogens (Angulo *et al.*, 2000; UCS, 2001; Molbak, 2004). The World Health

**Table 4.** Bivariate correlation analysis between  $tet^R$  and measured parameters (excluding individual resistance genes) for the eight feedlot lagoons.

	Positive correlation	Negative correlation
$P < 0.01$	Lot size (0.67) 16S-rRNA gene <sup>a</sup> (0.51) Tetracycline <sup>a</sup> (0.38) Head count (0.36) VSS (0.35)	Length of day (−0.40) Sun hours (−0.39)
$P < 0.05$	TSS (0.28)	pH (−0.35) Water temperature (−0.32)

a. Values were log-transformed to improve population distribution (normality) prior to correlation analysis. Brackets note Pearson Correlation Coefficients.



**Fig. 2.** Tetracycline resistance gene copy numbers over time for *tet(O)*, *tet(W)*, *tet(Q)*, *tet(M)*, *tet(L)* and *tet(B)*. Absolute gene copy numbers are presented for (A) no-use, (B) mixed-use and (C) high-use feedlot lagoons, whereas copy numbers normalized to ambient 16S-rRNA gene level are presented for (D) no-use, (E) mixed-use and (F) high-use feedlot lagoons respectively. Note that the Y-axis scales for absolute gene copy numbers and normalized gene copy numbers for the No-use lagoons (A and D) are two and three orders of magnitude lower respectively, than the mixed-use and high-use lagoons (B, C, E and F).

Organization recently called for the elimination of antibiotics for growth promotion in agriculture that also is used in medicine (WHO, 2004) and, in fact, the European Union banned antibiotics for growth promotion in early 2006 (UCS, 2006). Much of the work that led to these conclusions focused on resistance transfer through the food supply. However, resistance might also migrate away from operations via water, and this study was performed to examine relationships between on-site antibiotic use and resistance gene abundances below CAFOs.

Eight feedlot lagoons were monitored for 6 months to quantitatively compare tetracycline resistance gene levels in lagoons and local antibiotic use. Figure 1A shows significantly higher elevated resistance gene levels (*tet*<sup>R</sup>) in mixed-use and high-use feedlot lagoons with two to three orders of magnitude greater *tet*<sup>R</sup> levels than no-use feedlots. Although 16S-rRNA gene levels (i.e. total bacterial community sizes) were greater in the higher-use lagoons, Fig. 1B indicates that relative resistance gene levels were also greater when antibiotic use was greater. This general trend was confirmed by bivariate correlation analysis on all measured parameters (Table 4), which showed that conditions often associated with higher-use operations were most correlated with high resistance gene abundances.

Interestingly, correlation analysis also showed that tetracycline levels in the lagoons were only weakly correlated with *tet*<sup>R</sup> levels, although higher tetracycline levels were consistently seen in the water columns of the higher-use lagoons (Table 3). This weak correlation probably results either from differing environmental fate and transport mechanisms of resistance genes versus tetracycline after release (Doi and Stoskopf, 2000; Tolls, 2001) or,

possibly, the localization of tetracycline (or resistance genes) in lagoon sediments. Unfortunately, tetracycline(s) was only monitored in the water column (because the primary purpose of the analysis was to verify claimed use-patterns at each site); therefore, neither hypothesis can be proven with certainty.

Although these results are not surprising, they clearly demonstrate a strong relationship between on-site antibiotic use and resistance gene abundances in surface waters at the field-scale. The most significant relationship between actual 'on-the-ground' practices and observed gene levels was seen in the high-use lagoons in the autumn (Fig. 2). As background, tetracycline(s) are used for two main purposes in large cattle feedlots in the Midwestern United States: for the treatment of sick animals and as a preventative measure against the transmission of respiratory infection via cattle moving from one herd to another. Treatment of sick animals occurs year round, whereas preventative applications tend to be more seasonal. Specifically, a 5 day tetracycline dosing program is very common for newly weaned calves in the autumn when such calves are being moved to new herds. This practice, which primarily occurs at higher-use facilities, is very apparent in Fig. 2 data that show dramatically increased *tet*<sup>R</sup> levels in the high-use lagoons at that time (Fig. 2B, C, E and F).

Fortunately, all runoff at our sites was buffered by lagoons; however, without the lagoons, a substantial release of resistance genes would have occurred in the autumn with possible public health implications. As such, our data argue for (i) lagoons at feedlots as a minimum practice, (ii) restricted use of lagoon effluents for irrigation on crops without consideration of recent feedlot activity

and (iii) reduced use of tetracycline where effluents are released to surface waters. The second recommendation is particularly noteworthy because it has major implications in the possible seasonal transfer of resistance genes via water to the food supply that may have been missed through less rigorous monitoring.

An ancillary observation from this study is that different resistance genes appear to prevail in different lagoons according to season (even lagoons that exclusively service cattle). In theory, prevalent resistance genes in a given lagoon should reflect the enteric flora of the involved animal, the type of animal, terrestrial and aquatic conditions between the animal and the lagoon, and the tendency of different genes (and/or their hosts) to survive after release into the environment. Although Fig. 1 shows that *tet(O)*, *tet(Q)*, *tet(W)* and *tet(M)* were all higher in both absolute and relative abundance at feedlots with higher tetracycline use, Fig. 2 shows that *tet(M)* was the dominant gene in the high-use lagoons in the autumn when tetracycline was used most frequently. Given that *tet(M)* was not enriched in the no-use lagoons, *tet(M)* would appear to be a possible biomarker for 'high' use downstream of cattle-associated CAFO facilities.

The relatively high level of *tet(M)* is not surprising because it is seen equally in both Gram(+) and Gram(-) bacteria, and it is the most commonly detected gene among bacteria studied thus far (Roberts, 2005). The lack of *tet(B)* is also not surprising because it is more associated with Gram(-) bacteria, which are less common in cattle fecal matter (usually ~15–20%; Tajima *et al.*, 1999; Ozutsumi *et al.*, 2005). However, the lack of *tet(L)* was not expected because it is more often linked with Gram(+) bacteria and one would have expected *tet(L)* to be higher in cattle wastes. Whether the higher level of *tet(M)* or the lack of *tet(L)* or *tet(B)* is related to in-animal ecology or differences in gene migration or decay patterns after release is not known. However, these observations suggest that resistance genes in environments downstream of CAFO cattle operations is sometimes predictable, but not always, which has also been seen in soils at swine feedlot facilities (Schmitt *et al.*, 2006).

In summary, antibiotic use at CAFOs clearly influences the absolute level of resistance genes (important for predicting actual gene fluxes to the environment) and the proportion of resistance genes relative to the whole bacterial community (important for understanding the ecology of resistance in a community) in lagoons. Although work is still needed to understand the implications of these results, the product of this observation is already apparent. One of the high-use feedlots in this study that had been using lagoon water for crop irrigation has now reduced this practice, especially in the autumn. This feedlot has also reduced the amount of antibiotics it uses and is considering redesigning their lagoons to permit

safer irrigation. This anecdote shows that operators can modify their behaviour when provided strong evidence and, as such, we suggest that more practical work be performed on resistance genes in the environment to further demonstrate the value of more environmentally friendly practices at CAFOs.

## Experimental procedures

### Study sites – feedlot lagoons

Eight lagoons located at five cattle feedlots located across the Midwestern United States were sampled from June 2003 to November 2003. The feedlots and lagoons were assigned generic numbers to provide confidentiality to cooperating operators in the study. These feedlots and lagoons were carefully chosen to reflect different tetracycline use patterns while providing as much consistency as possible in the size and shape of the lagoons, and local drainage patterns. Table 1 provides a general description of the feedlots and their lagoons; the lagoons are classified according to their historical tetracycline use (as mentioned previously in *Results*).

### Field sampling and sample processing

Field measurements of temperature, pH and dissolved oxygen (DO) were performed in each lagoon using a Water Checker Field Monitor (Horiba Instruments). Typically, the probe was inserted to a 0.5 m depth at the same location during each visit, although the probe was sometimes higher in the water column when lagoon water levels were low. Liquid samples were also collected during each sampling visit using a 1.2 m long, 25 mm diameter PVC tube sampler equipped with a check valve (Graham *et al.*, 1999). Liquid samples were obtained by wading into each lagoon to locations near the inlet, outlet and a pre-defined midpoint, and gently inserting the sampler into the water column to minimize sediment disturbance during sampling. Typically, three duplicate samples were collected from each lagoon that were combined in the field to create two composite samples for analysis from each sampling. The composite samples were stored in 1 l, pre-sterilized amber glass bottles and retained in the dark on ice until return to the laboratory for final processing (< 24 h of sample collection).

Upon return to the laboratory, three 2 ml aliquots were collected from each composite sample and centrifuged at 20 000 *g* for 10 min. Supernatants were decanted and the resulting pellets were frozen at -80°C for DNA extraction. The remaining portion of each composite sample was stored at 4°C for other analysis, including tetracycline and TSS/VSS analysis (see Smith *et al.*, 2004 and APHA *et al.*, 1998), which was always performed < 48 h after sample collection.

### Analytical procedures

*Real-time PCR for quantification of resistance genes.* DNA was extracted according to protocols specified for DNA isolation from stool samples using the QIAamp® DNA Stool Mini

**Table 5.** Primers and TaqMan probes used in this study.

Target <sup>a</sup>	Sense primer	TaqMan probe <sup>b</sup>	Anti-sense primer
16S-rRNA gene <sup>c</sup>	ATGGCTGTCGTCAGCT	CCAAAGTACCACCATACGCAG	ACGGGCGGTGTGTAC
<i>tet</i> (B)	ACACTCAGTATTCCAAGCCTTTG	AAAGCGATCCCACCACCAGCCAAT	GATAGACATCACTCCCTGTAATGC
<i>tet</i> (L)	GGTTTTGAACGTCTCATTACCTGAT	CCACCTGCGAGTACAAACTGGGTGAAC	CCAATGGAAAAGGTTAACATAAAGG
<i>tet</i> (M)	GGTTTCTCTTGGATACTTAAATCAATCR	ATGCAGTTATGGARGGGATACGCTATGGY	CCAACCATAYAAATCCTTGTTTCRC
<i>tet</i> (O) <sup>d</sup>	AAGAAAACAGGAGATTCCAAAACG	ACGTTATTTCCCGTTTATCACGG	CGAGTCCCAGATTGTTTTTAGC
<i>tet</i> (Q) <sup>d</sup>	AGGTGCTGAACCTTGTGTTGATTC	TGCATCAGCATCCCGCTC	GGCCGGACGGAGGATTT
<i>tet</i> (W) <sup>d</sup>	GCAGAGCGTGGTTCAGTCT	TTCGGGATAAGCTCTCCGCCGA	GACACCGTCTGCTTGATGATAAT

a. Sequences denoted 5'–3'.

b. Sequence modifications added: 5'-FAM (6-carboxyfluorescein; fluorophore); 3'-TAMRA (carboxytetramethylrhodamine; quencher).

c. Based on Harms and colleagues (2003).

d. Based on Smith and colleagues (2004).

Kit as described previously (Smith *et al.*, 2004). Probe/primer sets for *tet*(O), *tet*(W), and *tet*(Q) were reported previously (Smith *et al.*, 2004), whereas probe/primers for *tet*(M), *tet*(L) and *tet*(B) were newly designed using Primer Express software. Probe/primer sets for 16S-rRNA genes were adapted from Harms and colleagues (2003). Specific probe/primers are summarized in Table 5 and were purchased from Sigma-Genosys (The Woodlands, TX). Standards for the different genes were constructed by transforming gene-bearing plasmids into *Escherichia coli* and purifying each as positive controls. Copy numbers for each gene were quantified against standards, ranging from  $1 \times 10^7$  to 1.0 copies ml<sup>-1</sup>, prepared by serial dilution of purified plasmid DNA. PCR amplifications were performed in duplicate using an ABI 7700 Sequence Detection System or Bio-Rad I-Cycler Detection System, and Taqman Universal PCR Master Mix (Smith *et al.*, 2004).

**Tetracycline quantification.** Free tetracycline was determined using the RIDASCREEN® ELISA tetracycline detection kit (R-Biopharm, Darmstadt, Germany). This ELISA is a class-specific assay that detects various tetracycline derivatives and is a good general measure of ambient tetracycline level in the water column (Aga *et al.*, 2003), which was our goal. Typically, 10 ml of lagoon water was centrifuged at 3000 *g* for 10 min. The resulting supernatant was decanted and diluted at a 1:10 ratio (50 µl to 450 µl) with sample buffer in glass test tubes, although a 1:50 ratio (50 µl to 2450 µl) was used for higher tetracycline samples.

A subset of samples was analysed using liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS; Kim *et al.*, 2005). This analysis was useful to verify samples with tetracycline levels near the ELISA detection limit (< 0.5 µg l<sup>-1</sup>) because experience indicated that the ELISA generates false-positive signals when tetracycline levels are lower. LC-ESI-MS provided the additional benefit of detecting other tetracyclines, including oxytetracycline, tetracycline, demeclocycline, chlortetracycline, doxycycline and anhydrochlortetracycline (not shown). LC-ESI-MS detection limits ranged from 0.3 to 0.8 µg l<sup>-1</sup> depending on the compound.

**Local weather conditions.** Weather data near each lagoon were obtained from the National Climatic Data Center main-

tained by the National Oceanic and Atmospheric Administration (NOAA, 2004). Reported data were from weather stations within 20 miles of each lagoon, and data were averaged and sorted over 4 week windows centring on each sampling day. This approach did not generate precise weather data at each lagoon, but provided an estimate of local weather trends around each sampling day. The total sunlight hours were obtained from the United States Naval Observatory (USNO, 2004).

#### Data analysis

**Estimation of the water conditions in the lagoons.** Estimated mean physical/chemical conditions in each lagoon were based on 8–10 sample days over the sampling period. Duplicate samples were collected on each day, therefore reported sample means were based upon between 16 and 20 samples per lagoon. Physicochemical data means from lagoons associated with each antibiotic use strategy were also combined to estimate mean lagoon water conditions for each type of feedlot lagoon ( $n \approx 54$ ). Confidence intervals (CI; 95%) were determined from standard deviations associated from each estimated mean.

Mean real-time PCR gene numbers and tetracycline levels were calculated similar to physicochemical lagoon water conditions with the following exception. It was observed that the ELISA sometimes indicated tetracycline in samples where tetracycline was not likely present. Therefore, all near-detection-limit ELISA estimates (< 2.0 µg l<sup>-1</sup>) were re-analysed using LC-ESI-MS analysis, and LC-ESI-MS data were substituted into the dataset for the low tetracycline samples. LC-ESI-MS was also performed on a subset of higher tetracycline samples to verify that data substitution did not bias the dataset, and it was found that LC-ESI-MS and the ELISA significantly correlated at tetracycline levels > 2.0 µg l<sup>-1</sup> ( $r = 0.93$ ,  $P < 0.01$ ).

**Comparison among lagoons and antibiotic use strategies.** The Kruskal–Wallis test was used to compare water chemistry conditions, free tetracycline levels, and resistance gene levels among lagoons and feedlots with different antibiotic use patterns. This is a non-parametric test for determining whether data come from the same or differ-

ent parent populations (Kanji, 1999). Pearson's bivariate correlation analysis was also used to assess trends among tet<sup>R</sup> and other parameters monitored (SPSS, 2001). Resistance gene numbers were usually not normally distributed; therefore, all gene data were log-transformed prior to statistical analysis.

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